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Evaluation of cytotoxic and mutagenic effects of two artificial sweeteners by using eukaryotic test systems

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Considering the vast use of sweeteners by the Brazilian population and the constant need for toxicological studies of food additives, this study aimed to evaluate the cytotoxic and mutagenic potentials of the sodium saccharin and/or sodium cyclamate sweeteners in plant (*Allium cepa)* and animal (*Mus musculus*) test systems based on concentrations permitted by the Brazilian laws. In *A. cepa*, both the sweeteners individually and their combinations concentration and exposure time (ET: 48, 72 and 168 h) dependently exerted cytotoxic and mutagenic effects. Similarly, an increased in micronuclei formation was also observed by the sweeteners in peripheral blood cells in mice. More toxic effects were observed with the combination doses at 168 ET. There may be a synergistic effect and DNA damage with an increasing concentration and ET. In conclusion, the concentrations considered safe by the Brazilian laws had significant cytotoxic and mutagenic activity on the eukaryotic cells.

Key words: *Allium cepa*, *Mus musculus*, sweeteners, toxicity.

INTRODUCTION

Food additives are the substances intentionally added to food without the nourishing purpose. In Brazil, the governing rules of the use of food additives are controlled by the National Health Surveillance Agency (ANVISA)

based on the international benchmarks, such as the Codex Alimentarius, the European Union and, complementarily, the US Food and Drug Administration (ANVISA, 2009). Sweeteners are the food additives that are added with technological or organoleptic intention to give the sweet taste without adding calories at any stage of food processing (Jain and Grover, 2015).

Among the sweeteners accepted for the use by ANVISA, sodium saccharin and sodium cyclamate stand out. Saccharin was introduced in 1878, which is 300 times sweeter than the sucrose, and not metabolized in the human body. It is synthesized from the toluene sulfonic acid, derived from petroleum (Pearson, 2001). In aqueous solution, it may be used in a conjunction with other sweeteners (Fitch and Keim, 2012) such as salts of cyclamic acid or cyclamates. These acids are prepared using chlorosulfonic acid and were discovered by Sveda and Audrieth (Jain and Grover, 2015).

In recent decades, the consumption of these types of sweeteners has been increased. It may be due to their synergistic sweetening properties and improved taste of the product. Dietetic products (especially diet sodas and diet sweeteners) have been the main sources of intake of non-caloric sweeteners (Alzin, 2003). However, there is no consensus on the safety of the use of cyclamate and saccharin. Studies on the toxicologic and carcinogenic potential of these substances are still quite controversial. There are studies that show that, these sweeteners cause testicular atrophy with an increase in the incidence of bladder tumors in mice. Although, there is no evidence in human risks, but animal studies have encouraged the prohibition of these sweeteners in some countries, such as Canada, USA, England, France and Japan (Uçar and Yilmaz, 2015; Mishra et al., 2015).

Despite all this controversy, the use of sodium cyclamate and sodium saccharin is authorized in more than 50 countries. Brazil is one of them (ASAE, 2006; CODEX, 2007). The last toxicological evaluation of sweeteners was performed by JECFA in 1982, following a setting of a safety limit (JECFA, 2000). However, in Brazil, ANVISA has not conducted any study related to safety concentrations other than allowing a considerable limits use in foods; thus, the essence of conduction of the toxicological studies on these types of sweeteners.

Bioassays with plants and animals have been considered highly sensitive and simple in monitoring cytotoxic and mutagenic effects of food additives (Iganci et al., 2006). Among them, *Allium cepa* is an excellent test system to indicate possible toxicological effects of a wide variety of substances. It is popularly used due to the fact that the cells in the meristematic region of roots have kinetic properties of proliferation and have a small number of chromosomes (2n = 16), leading to raise reliability and agreement with the other toxicological studies using higher eukaryotic test systems (Tabrez et al., 2011).

On the other hand, among the genotoxicity assessment tests recommended by the international agencies and government health institutions, the micronucleus test in rodents' peripheral blood is widely accepted (Ribeiro et al., 2003), which allows identification of increased mutation frequency exposed to a particular genotoxic agent for short or long terms (Yadav et al., 2014).

By considering the above mentioned facts, this study aimed to evaluate the cytotoxic and mutagenic effects of the above mentioned sweeteners using *A. cepa* and *Mus musculus* test systems.

MATERIALS AND METHODS

Selection and preparation of test concentrations

The concentrations of the tested sweeteners were calculated in accordance with the limits set by ANVISA through the law (RDC) No. 18 on March 24, 2008. The maximum allowed values for each sweetener were 100 g or 100 ml of ready-to-eat food. Based on the legislation, we have defined three concentrations of each food additive in this study as shown in Table 1. The exposing effect in both system tests was based on the maximum daily consumption level considered safe by ANVISA.

A. cepa assay

A. cepa assay in this study was done according to the method described by Islam et al. (2016). Briefly, small, uniform, same origin, not germinated and healthy onions was used. The onions were placed in vials containing distilled water at room temperature for rooting. Then the onions (5/each concentration) with multiple roots at least 0.5 cm of each, were selected for the treatment solutions. The exposure time (ET) was set at 48, 72 and 168 h. Copper sulfate (0.0012 g/L) was used as a standard in this test. After the elapsed ET, the root tips (meristem) were removed and fixed in Carnoy (3: 1 ethanol: acetic acid) solution. For photomicroscopic analysis, the cells were harvested and stained with 2% acetic orcein. Slides were evaluated using an optical microscope (100X), where 1000 cells were evaluated per repetition (5,000 cells per treatment). The mitotic index (MI) and chromosomal aberrations (CA) were evaluated to determine the cytotoxic and mutagenic effects, respectively.

MN assay in mice

In this purpose, male *Swiss* mice (30-35 g body weight, 2 months old) were obtained from the animal facility of the Federal University of Piauí (UFPI, Piauí / Brazil) and acclimatized under standard environmental conditions (12 h light/dark cycle and temperature of 23 ± 2°C) with free access to food and drinking water *ad libitum*. The experimental protocol was approved by the Ethics Committee for Animal Research Experimentation (CEEA) of the Federal University of Piauí, Brazil (No. 156/2016).

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Table 1. Tests and concentrations tested for sodium saccharin and/or sodium cyclamate and the test systems.

Treatments	Test systems	
	Allium cepa	***Swiss* mice**
Negative control	Distilled water	
Sodium saccharin	10 mg/ 100 g/ml 15 mg/ 100 g/ml 30 mg/ 100 g/ml	
Sodium cyclamate	20 mg/ 100 g/ml 40 mg/ 100 g/ml 80 mg/ 100 g/ml	
Sodium saccharin + Sodium cyclamate	10 mg/100 g/ml sodium saccharin + 20 mg/100 g/ml sodium cyclamate (dosages below the limits permitted by ANVISA for daily consumption)	
	15 mg/100 g/ml of sodium saccharin + 40 mg/100 g/ml sodium cyclamate (maximum dosage allowed by ANVISA for daily consumption)	
	30 mg/100 g/ml of sodium saccharin + 80 mg/100 g/ml Sodium cyclamate (dosages above those limits permitted by ANVISA for daily consumption)	
Positive control	Copper sulfate 0.0012 g/L	Cyclophosphamide (5 mg/ 100 g/ml) equivalent to 36.4% of lethal dose (137 mg/kg)

A total of 55 mice was grouped accordingly, five for each concentration. The negative control (distilled water, 1 ml/ 100 g) and test samples were administered *via* oral gavage (0.5 ml), while the positive control (cyclophosphamide, 5 mg/ 100 g/ml) was given by an intraperitoneal (i.p.) injection. All the treatments were given once a day for 7 consecutive days. The peripheral blood was collected by the puncturing of the tail vein at the times of 48, 72 and 168 h after the treatment initiation. Collected blood was divided into two slides for each animal and followed by drying for 12 h at 22°C. The slides were then fixed in methanol P.A. and subsequently stained for 10 min in a solution of 3% Giemsa. A total of 2000 cells (1000 cells per slide) were observed for each concentration of the test and controls. Slides were evaluated using an optical microscope with 100X magnification (Taimo et al., 2016).

Statistical analysis

The results of the two tests were performed by the RM-MANOVA test, followed by Tukey post-test, using the program STASTISTIC 7.0, considering $p < 0.05$.

RESULTS

According to Table 2, it is clear that the PC and sodium saccharin and/or sodium cyclamate concentration and ET dependently decreased MI but increased in CA formation in *A. cepa* test. The NC did not show such type of regularity in the MI and CA values. The lowest MI (4.1 ± 1.1 at 168 ET) was observed at 40 mg/100 g/ml of sodium cyclamate, while highest CA (28.2 ± 2.5) with the combined treatment of sodium saccharin (10 mg/100 g/ml) and sodium cyclamate (20 mg/100 g/ml) group. However, more cytotoxic and mutagenic effects were attributed with the combined treatments with these sweetners. The standard copper sulphate at 168 ET exhibited the highest CA by 50.0 ± 1.0.

Table 3 depicts a MN profile with the treatments at ET 48, 72 and 168 h in the peripheral blood cells of mice. Here is also a concentration and time-dependent augmentation in MN values of the test samples and cyclophosphamide (CP). The highest MN (14.4 ± 3.6) was observed in the co-treatment group of sodium saccharin and sodium cyclamate (30 + 80 mg/100 g/ml) group at 168 ET. Sodium cyclamate at 168 ET was found to produce MN by 13.1 ± 3.1 at 80 mg/100 g/ml. The NC attributed a reduced number of MN at 72 h other than 48 and 168 h.

DISCUSSION

The sodium cyclamate and sodium saccharin with a ration of 2:1 are popularly used to mask the bitter taste of some products (Zanini et al., 2011). In 2001, ANVISA decided to reduce the maximum amount of these two sweeteners in drinks and foods. With this decision the RDC n. 3/2001 was repealed and the maximum cyclamate usage ranged from 97 to 130 mg to 40 to 56 mg per 100 ml or 100 g, while saccharin by 22 to 30 mg to 10 to 15 mg. This decision was made according to the raised concerns about the possible toxicological and neoplastic effects on the human body.

Table 2. Cytotoxicity and mutagenicity of sodium saccharin and sodium cyclamate in *Allium cepa.*

Treatments		ET 48 h		ET 72 h		ET 168 h	
		MI	CA	MI	CA	MI	CA
NC		15.1 ± 0.4	5.5 ± 1.5	13.0 ± 1.4	9.0 ± 2.5	15.87 ± 0.9	7.0 ± 0.5
PC (0.0012 g/L)		12.5 ± 1.6	26.0 ± 4.5[a]	7.2 ± 1.1[a]	33.5 ± 4.5[a]	6.9 ± 0.8[a]	50.0 ± 1.0[a]
Sodium saccharin (in 100 g/ml)	10 mg	9.2 ± 2.8[a]	6.7 ± 2.7	7.7 ± 1.5[a]	8.2 ± 3.2	5.62 ± 1.8[a]	11.7 ± 4.7
	15 mg	10.2 ± 0.9[a]	9.25 ± 2.8	7.2 ± 0.8[a]	10.0 ± 4.0	6.6 ± 2.5[a]	12.5 ± 2.5
	30 mg	10.8 ± 2.4	10.5 ± 4.5	6.8 ± 1.6[a]	12.5 ± 2.5	6.3 ± 0.8[a]	13.2 ± 2.2[a]
Sodium cyclamate (in 100 g/ml)	20 mg	11.5 ± 3.3	9.0 ± 3.9	9.3 ± 1.9	12.0 ± 1.8	5.9 ± 1.4[a]	13.7 ± 15
	40 mg	11.0 ± 4.8	9.2 ± 3.1	8.6 ± 1.5[a]	12.5 ± 8.1	4.1 ± 1.1[a]	16.7 ± 7.8[a]
	80 mg	7.7 ± 1.1[a]	7.2 ± 4.6	6.1 ± 2.7[a]	11.7 ± 3.1	5.4 ± 1.8[a]	14.2 ± 7.5[a]
Sodium saccharin + Sodium cyclamate (in 100 g/ml)	10 + 20 mg	13.5 ± 3.8	5.2 ± 1.5	5.57 ± 1.1[a]	19 ± 1.4[a,b]	5.07 ± 1.4[a]	28.2 ± 2.5[a,b]
	15 + 40 mg	8.9 ± 1.7[a]	18.5 ± 1.2[a]	5.4 ± 0.8[a]	19.7 ± 1.7[a,b]	4.3 ± 1.7[a]	21.2 ± 1.6[a,b]
	30 + 80 mg	15.4 ± 2.6	16.07 ± 2.1[a]	6.0 ± 0.6[a]	16.2 ± 0.9[a,b]	5.1 ± 0.4[a]	19.2 ± 2.6[a]

MANOVA with Tukey *post-test*. ET, exposure time; NC, negative control (distilled water); PC, positive control (Copper sulfate 0.0012 g/L); MI, mitotic index; CA, chromosomal aberrations; [a]p <0.05 compared to the NC (same TE analyzed); [b] p <0.05 compared to the individual sweeteners in the same concentration and ET.

Table 3. Micronuclei formation by the sodium saccharin and sodium cyclamate in mice.

Treatments		MN		
		ET 48 h	ET 72 h	ET 168 h
NC (1 ml/ 100 g)		3.7 ± 1.6	3.2 ± 1.8	2.8 ± 1.6
PC (5 mg/ 100 g/ml)		10.3 ± 2.6[a]	10.6 ± 1.8[a]	11.9 ± 3.3[a]
Sodium saccharin (in 100 g/ml)	10 mg	1.4 ± 1.6	4.1 ± 1.7	8.5 ± 2.5[a]
	15 mg	3.3 ± 1.2	3.7 ± 1.6	8.9 ± 2.9[a]
	30 mg	3.2± 1.1	3.3 ± 2.1	7.3 ± 1.4
Sodium cyclamate (in 100 g/ml)	20 mg	2.8 ± 1.7	4.8 ± 2.1	5.9 ± 1.6
	40 mg	3.9 ± 2.2	9.0 ± 1.4[a]	9.1 ± 1.7[a]
	80 mg	3.2 ± 1.6	5.4 ± 1.1	13.1 ± 3.1[a]
Sodium saccharin + sodium cyclamate (in 100 g/ml)	10 + 20 mg	5.7 ± 3.1	5.8 ± 1.8	6.0 ± 2.1
	15 + 40 mg	8.6 ± 2.6	8.6 ± 2.1[a]	9.2 ± 2.4[a]
	30 + 80 mg	13.1 ± 3.2[a,b]	14.0 ± 3.2[a]	14.4 ± 3.6[a,b]

RM, MANOVA with Tukey *post-test*. ET, exposure time; MN, micronuclei; NC, negative control (distilled water); PC, positive control (cyclophosphamide, 5 mg/100 g/ml); AC, chromosomal aberrations; [a]p <0.05 compared to the NC (same ET analyzed); [b]p <0.05 compared to the individual sweetener in the same concentration and ET.

In the earlier studies, it has been shown that these two sweeteners have genotoxic effects on a number of test systems (Bandyopadhyay et al., 2008). Before that, Sasaki et al. (2002) evaluated five sweeteners, including these two using comet assay in cells of various organs of rats exposed for 3 and 24 h of treatment revealed that the sodium cyclamate induced an increase in DNA damage in the glandular cells of the stomach, colon, kidney, and bladder. The authors also reported saccharin-induced alterations in the glandular cells of the stomach and colon even at the lowest concentration tested (1000 mg/kg). In this study, in *A. cepa* test, a concentration and ET-dependent alterations of MI and CA values in the sodium cyclamate and/or sodium saccharin groups was also found. More alterations were observed in the combination group of the sweeteners at 168 h. However, the activity was lower than the PC group.

Furthermore, an increased risk of bladder cancer (30% of experimental animals treated with a dose of 7.5% of sodium saccharin in their diet) was consistently proven to the second generation of rodents with these sweeteners (Weihrauch and Diehl, 2004). In the US, since 1981, products containing saccharin are needed to be labeled with a warning that saccharin may cause cancer in

laboratory animals. Moreover, cyclohexylamine, a metabolite of cyclamate is evident to exert toxic effects on the animals, possibly due to its cytotoxic and mutagenic effects (Bastaki, 2015). In a study, cyclohexylamine caused testicular atrophy and reduced spermatogenesis in rats and dogs (Weihrauch and Diehl, 2004). Additionally, Martins et al. (2010) found a late fetal development and pancreas hypertrophy in rat fetuses exposed to sodium cyclamate.

A study conducted by Van Eyk (2015) found that aspartame, sodium cyclamate, acesulfame K and sodium saccharin are cytotoxic, mutagenic and genotoxic to Caco-2 cell lines (colonic cells), HT-29 (colon cells) and HEK-293 (renal cell). These results corroborate with those of Sasaki et al. (2002) that used the comet assay and reported that sodium saccharin and sodium cyclamate exerted genotoxic and mutagenic effects on rodent colon cells. Demir et al. (2014) also suggested that, both acesulfame K and sodium saccharin may induce DNA damage in rodents. A case-control study by Andreatta et al. (2008), demonstrated that the incidence of tumors of the urinary tract and the consumption of sweeteners in Cordoba in Argentina. Our study conducted on *Swiss* mice, suggesting that both sodium saccharin and/or sodium cyclamate has mutagenic effects in peripheral blood lymphocytes. Both of them with or without combining treatments augmented a concentration and ET-dependent MN production in the test systems. In some cases, we have seen that the middle concentration/dose exerted more cytotoxic or mutagenic effects in comparison to the lowest and highest treated concentration/dose. It may be due to the adaptation responses in the test systems.

Conclusion

Sodium saccharin and sodium cyclamate sweeteners showed cytotoxic and mutagenic effects both in vegetal (*A. cepa*) and animal (*Swiss* mice) cells at the concentrations allowed by the Brazilian law. More toxic effects were observed with their combined treatments. There may be a synergistic effect. Further studies are urgently needed, using more intensive bioassays, such as human cell lines, to say exact mechanisms of cytotoxic, genotoxic and mutagenic effects of these popularly used artificial sweeteners.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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